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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY DOCKET NO.
P07374US00/LRP

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
09/937035

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/00725	22 MARCH 2000	23 MARCH 1999

TITLE OF INVENTION: METHOD FOR DETECTING PREDISPOSITION TO A VENOUS THROMOEMBOLIC . . .

APPLICANT(S) FOR DO/EO/US: EMMERICH, Joseph et al.

Applicant herewith submits to the US Designated/Elected Office (DO/EO/US) the following items and other information:

☒ 1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

☐ 2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.

☒ 3. This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Art. 22 and 39(1).

☒ 4. A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

☒ 5. A **copy** of the International Application as filed (35 U.S.C. 371 (c)(2))

- ☐ a. is transmitted herewith (required only if not transmitted by the International Bureau).
- ☒ b. has been transmitted by the International Bureau.
- ☐ c. is not required, as the application was filed in the United States Receiving Office (RO/US).

☐ 6. A **translation** of the International Application into English (35 U.S.C. 371(c)(2)).

☒ 7. Amendments to the claims of the International Appln. under PCT Article 19 (35 USC 371 (c)(3))

- ☐ a. are transmitted herewith (required only if not transmitted by the International Bureau).
- ☐ b. have been transmitted by the International Bureau.
- ☐ c. have not been made; however, the time limit for making such amendments had NOT expired.
- ☒ d. have not been made and will not be made.

☐ 8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

☐ 9. An **oath** or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

☐ 10. A translation of the annexes to the Int'l Prelim. Exam. Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

☐ 11. An **Information Disclosure Statement** under 37 C.F.R. 1.97 and 1.98.

☐ 12. An **Assignment** document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

☐ 13. A **First preliminary amendment**.

☐ 14. A Second or Subsequent preliminary amendment.

☐ 15. A substitute specification.

☐ 16. A change of power of attorney and/or address letter.

☐ 17. A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 & 35 USC 1.821-825.

☐ 18. A second copy of the published international application under 35 USC 154(d)(4).

☐ 19. A second copy of the English translation of the international application under 35 USC 154(d)(4).

☐ 20. Other items or information:

☐

☐

☐ A copy of the Notification of Missing Requirements under 35 U.S.C. 371.

☐ In the event that a petition for extension of time is required to be submitted herewith, and in the event that a separate petition does not accompany this response, applicant hereby petitions under 37 CFR 1.136(a) for an extension of time of as many months as are required to render this submission timely. Any fee is authorized in 17(c).

Date: 21 September 2001

LARSON & TAYLOR, PLC • 1199 North Fairfax St. • Suite 900 • Alexandria, VA 22314

U.S. APPLICATION NO. (if known)	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NO.
09/937035	PCT/FR00/0072	P07374US00/LRP

☒ 21. **The following fees are submitted:**

		CALCULATIONS *PTO USE ONLY*
☒ **Basic National Fee** (37 CFR 1.492 (a) (1)-(5):		
☐ Neither Int'l Prelim. Exam. fee nor Int'l Search fee paid to USPTO	$1000	
☒ Search Report has been prepared by the EPO or JPO	$ 860	
☐ No Int'l Prelim. Ex. fee paid to USPTO but Int'l Search fee paid to USPTO	$ 710	
☐ International preliminary examination fee paid to USPTPO	$ 690	
☐ Int'l Prelim. Ex. fee paid to USPTO & all claims satisfied PCT Art. 33(1)-(4)	$ 100	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 860
☐ Surcharge of **$130** for furnishing the oath or declaration later than ☐ 20 mos. ☐ 30 mos. + from the earliest claimed priority date (37 CFR 1.492(e)).		$

CLAIMS	*NUMBER FILED*	*NUMBER EXTRA*	*RATE*	
Total Claims	- 20 =		X **$18** =	$
Independent Claims	- 03 =		X **$80** =	$
☐ Multiple Dependent Claim(s) (if applicable)			+ **$270** =	$
TOTAL OF ABOVE CALCULATIONS =				$
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.			-	$
SUBTOTAL =				$ 860
☐ Processing fee of **$130** for furnishing the **English translation** later than ☐ 20 mos. ☐ 30 mos. + from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 860
☐ Fee for recording the enclosed **assignment** (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40** per property			+	$
TOTAL FEES ENCLOSED =				$ 860
Amount to be				*Refunded* $
				Charged $

☒ a. A **check** in the amount of $ 860 to cover the above fees is enclosed.

☐ b. Please charge my Deposit Account No. 12-0555 in the amount of $ to cover the above fees.

☒ c. The Commissioner is hereby authorized to charge any additional fees required or credit overpayment to Deposit Account No. 12-0555.

Note: *Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.*

SEND ALL CORRESPONDENCE TO:

LINDA R. POTEATE

At the address (below) of **CUSTOMER NO. 00881.**

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Date: 21 September 2001

METHODS AND COMPOSITIONS FOR PREVENTING OR RETARDING THE DEVELOPMENT OF ATHEROSCLEROTIC LESIONS

Field of the Invention

The present invention relates generally to pharmaceutical compositions and methods of use thereof in treating or preventing atherosclerosis.

Background of the Invention

Atherosclerosis (AT) results from an excessive inflammatory and fibroproliferative response to vascular insult and has been noted to be a principal cause of heart attack and stroke, accounting for up to half of all mortalities in the industrialized world including about 13 million Americans. Atherogenesis is theorized to follow a response to injury, but the agent(s) of injury have yet to be identified fully.

Viral and/or bacterial infection(s) have been found to be associated in some way with the complex process of the development of AT. Particles, antigens and DNA sequences of human cytomegalovirus (HCMV), a member of the herpesvirus group, have been described in AT plaques of biopsy or autopsy material [M. G. Hendrix *et al*, Am. J. Pathol., 136:23-28 (1990); J. L. Melnick *et al*., BioEssays, 17(10): 899 (October 1995)]. However, Melnick, cited above, states that the "observations do not demonstrate a viral role in the pathogenesis of atherosclerosis". The possible involvement of reactivated HCMV in restenosis of coronary arteries, an accelerated form of AT, following angioplastic surgery has been suggested [S. E. Epstein *et al*, Lancet, 348:13-17 (1996); E. Speir *et al*, Science, 265:391-394 (1994); Y. F. Zhou et al, N. Engl. J. Med., 335: 624-630 (1996)]. Seroepidemiologic data show that HCMV infection usually occurs in childhood, paralleling the pattern of the appearance of early AT lesions; by young adulthood, 50-100% of individuals are HCMV-seropositive. In some

individuals, the virus is apparently reactivated in artery walls, where it may initiate abnormal cell growth that can lead to blocked blood flow and, ultimately, heart attack.

The bacterium *Chlamydia pneumoniae* is an intracellular bacterium, which has been established as an important pathogen in acute and chronic respiratory infections [J. T. Grayson, Clin. Infect. Dis., 15:757-763 (1992)] has also been associated with AT [J. A. Ramirez, Ann. Intern. Med., 125:979-982 (1996)]. This bacterium infects about 50% of the population and causes flu-like diseases, but also replicates in the arterial wall. *C. pneumoniae* antigens and DNA have been detected in human AT plaques. Population antibody prevalence studies have shown that more than 50% of adults worldwide have antibody. While antibody is infrequent in children under age 5 years, incidence studies have demonstrated antibody conversion of 6-9% per year in children from the ages 5-14 years. The prevalence of antibody continues to increase throughout adulthood, and is highest in the elderly.

Recently, *C. pneumoniae*, strain TWAR, has been associated with AT based on both seroepidemiology and data demonstrating the presence of the organism in AT plaques. For example, serologic studies from Finland, the United States and other countries have shown that patients with coronary artery diseases were significantly more likely to have serologic evidence of past infection with TWAR than were controls. Morphologic and microbiological evidence of the persistence of TWAR in atheromatous plaques has been obtained by electron microscopic studies, immunochemical staining and PCR testing of coronary, carotid and aortic atheroma [C.-C. Kuo *et al*, Clin. Microbiol. Rev., 8:451-461 (1995)]. In addition, *C. pneumoniae* activates growth factors involved

in inflammatory responses and changes lipoprotein metabolism of infected cells. Immune responses to chlamydial infections are partly protective but also deleterious, and delayed hypersensitivity (DH) is thought to play a pathogenic role in chlamydial disease [R. P. Morrison *et al*, J. Exp Med., 170:1271-1283 (1989)].

Despite the wealth of reports, no etiological role of HCMV and/or *C. pneumoniae* in the development of AT has been established. Thus, there remains a need in the art for reagents and methods useful in ameliorating the symptoms and development of atherosclerosis in response to these microorganisms.

Summary of the Invention

In one aspect, the invention provides a method for the treatment or prophylaxis of atherosclerosis in a mammal, preferably a human, comprising administering to a mammal an effective amount of a composition comprising a human cytomegalovirus (HCMV) protein or fragment thereof, the amount capable of inducing cell mediated immunity and anti-CMV antibody response in the mammal. The composition preferably may be administered to infants or immunocompromised patients.

In another aspect the invention provides a method for the treatment or prophylaxis of atherosclerosis in a mammal comprising administering to a mammal an effective amount of a composition comprising a nucleic acid sequence encoding a human cytomegalovirus (HCMV) protein or fragment thereof, said composition capable of inducing cell mediated immunity (CMI) and inducing an anti-CMV antibody response upon expression of said protein in the mammal.

In still another aspect, the invention provides a method for the treatment or prophylaxis of atherosclerosis in a mammal, preferably a human,

comprising administering to a mammal an effective amount of a composition comprising a *Chlamydia pneumoniae* protein or fragment thereof, the amount capable of inducing cell mediated immunity and anti-*C. pneumoniae* antibody response in the mammal. The composition preferably may be administered to infants or immunocompromised patients.

In yet another aspect the invention provides a method for the treatment or prophylaxis of atherosclerosis in a mammal comprising administering to a mammal an effective amount of a composition comprising a nucleic acid sequence encoding a *C. pneumoniae* protein or fragment thereof, said composition capable of inducing cell mediated immunity (CMI) and inducing an anti-*C. pneumoniae* antibody response upon expression of said protein in the mammal.

In another aspect, the invention provides a therapeutic or prophylactic composition comprising a *Chlamydia pneumoniae* protein or fragment thereof and an HCMV protein or fragment thereof in a pharmaceutically acceptable carrier.

In still another aspect, the invention provides a therapeutic or prophylactic composition comprising an anti-microbial agent effective against *Chlamydia pneumoniae* infection and an HCMV protein or fragment thereof in a pharmaceutically acceptable carrier.

In yet another aspect, the invention provides a method for treating atherosclerosis or restenosis by administering to a mammal having physical evidence of atherosclerosis or restenosis an effective amount of an anti-microbial agent directed against *Chlamydia pneumoniae* infection.

In a further aspect, the invention provides a method for preventing restenosis after coronary atherectomy or balloon angioplasty comprising treating a patient prior

to, or after said atherectomy or angioplasty with an effective amount of said *C. pneumoniae*/HCMV composition described above or with an effective amount of an anti-microbial agent directed against *Chlamydia pneumoniae* infection.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1A is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a section of the aorta of an uninfected mouse. A lack of expression of viral antigens in the endothelial and smooth muscle cells in the aortic wall was detected by immunofluorescence (IF) assay using an anti-murine cytomegalovirus (MCMV) polyclonal mouse serum and FITC labeled second antibody (Chemicon), magnification X500.

Fig. 1B is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a section of aorta from the same mouse showing no accumulation of inflammatory cells in the aortic lumen. This is a parallel section stained with hematoxilin-eosin (HE), magnification X500.

Fig. 1C is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a parallel section of the aorta of the uninfected mouse as described in Fig. 1B, except that magnification was X1250.

Fig. 1D is a section of aorta from a mouse infected with the Smith strain of MCMV (ATCC Accession No. VR194), γ-irradiated (500 rad) and fed with a high cholesterol diet. This section shows the expression of viral antigens in the endothelial and smooth muscle cells in the aortic wall as detected by IF assay using anti-MCMV polyclonal mouse serum.

Fig. 1E is a section from the mouse of Fig. 1D, showing accumulation of inflammatory cells in the aortic lumen and in the periaortic area in a parallel section stained with HE, magnification X500.

Fig. 1F is a parallel section as described in Fig. 1E, except that magnification was X1250.

Fig. 1G is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a section of aorta from a mouse infected with the Smith strain of MCMV, γ-irradiated (500 rad) and fed with a normal mouse diet. This section shows the expression of viral antigens in the endothelial and smooth muscle cells in the aortic wall as detected by IF assay using anti-MCMV polyclonal mouse serum and FITC labeled second antibody (Chemicon), magnification X500.

Fig. 1H is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a section of aorta from the mouse of Fig. 1G, showing accumulation of inflammatory cells in the aortic lumen and in the periaortic area in a parallel section stained with HE, magnification X500.

Fig. 1I is a photograph demonstrating viral antigens and chronic inflammatory infiltrate in a parallel section of the aorta of the mouse described in Fig. 1H, except that magnification was X1250.

Fig. 1J is a section of aorta from a mouse infected with the Smith strain of MCMV, not γ-irradiated, and fed with a normal mouse diet. This section shows the expression of viral antigens in the endothelial and smooth muscle cells in the aortic wall as detected by IF assay using anti-MCMV polyclonal mouse serum.

Fig. 1K is a section from the mouse of Fig. 1J, showing accumulation of inflammatory cells in the aortic lumen and in the periaortic area in a parallel section stained with HE, magnification 1:630. A small area of

accumulation of inflammatory cells is seen in the right upper corner of the aorta.

Fig. 1L is a parallel section as described in Fig. 1K, except that magnification was X1575.

Fig. 2A illustrates LacZ expression and an early AT plaque in aorta of a MCMV-lacZ-infected mouse at magnification X500.

Fig. 2B is the photograph of Fig. 2A at magnification X1250.

Fig. 2C is the photograph of Fig. 2A at magnification X2500.

Fig. 3A illustrates *C. pneumoniae* antigens in the aorta of mice infected with *C. pneumoniae*, detected with IF, using a monoclonal antibody specific for the major outer membrane protein (MOMP), dilution 1:5 (DAKO Diagnostic Ltd., Great Britain) and Texas-red-labeled second antibody (Chemicon).

Fig. 3B illustrates an early AT plaque in the aorta of mice infected with *C. pneumoniae*, detected with H&E, magnification x1250.

Fig. 4A illustrates the expression of HCMV-IE antigens in human arterial smooth muscle cells, detected with IF, using the E13 monoclonal antibody, dilution 1:80 (Chemicon, USA) and FITC-labeled second antibody.

Fig. 4B illustrates the expression of *C. pneumoniae* antigens in human arterial smooth muscle cells, detected with IF, using monoclonal antibody MOMP, and Texas-red-labeled second antibody.

Detailed Description of the Invention

The present invention provides compositions and methods which are useful for both the treatment and prophylaxis of mammalian subjects against the onset or development of atherosclerosis or atherosclerotic lesions characteristic of restenosis or associated with other

arterial injury. The invention involves administering anti-microbial compositions directed against HCMV and *C. pneumoniae* to treat and/or prevent the development of atherosclerotic lesions and restenosis.

I. Compositions of the Invention

A. *CMV-containing Compositions*

The compositions useful according to this invention may contain a CMV protein or fragment. Thus, the composition can be an attenuated, live CMV, preferably HCMV. The composition may also be a heat-inactivated, attenuated HCMV. Vaccines based on live attenuated strains of HCMV have been described. [See, e.g., S. A. Plotkin et al, Lancet, 1:528-30 (1984); S. A. Plotkin et al, J. Infect. Dis., 134:470-75 (1976); S. A. Plotkin et al, "Prevention of Cytomegalovirus Disease by Towne Strain Live Attenuated Vaccine", in Birth Defects, Original Article Series, 20(1):271-287 (1984); J. P. Glazer et al, Ann. Intern. Med., 91:676-83 (1979); and U. S. Patent 3,959,466, all incorporated by reference herein.]

Alternatively, the composition may contain only certain HCMV proteins or fragments of these proteins which are characterized by the capacity to induce cell mediated immunity (CMI) to CMV infection upon administration of the protein in combination with an appropriate adjuvant. This composition may induce a low to undetectable anti-CMV antibody response in the subject. Desired HCMV proteins for this use include, without limitation, phosphoproteins 65, 150, 28 and 52, immediate early protein (IE), glycoprotein B (gB), and glycoprotein H (gH). Most desirably, the early HCMV proteins IE, gB and pp65 are useful in such a composition. One example of a useful fragment for this purpose is the immediate-early exon-4 (IE-exon-4) subunit

of the HCMV. [See, e.g., International Patent Application No. WO94/17810, published August 18, 1994, incorporated by reference herein].

Where the HCMV composition contains nucleic acid sequence(s) encoding a cytomegalovirus protein or fragment thereof, the nucleic acid sequence may be that of the selected protein itself, that is, a 'naked' DNA composition. So-called 'naked DNA' may be used to express the HCMV protein or peptide fragment *in vivo* under the control of suitable promoter sequences. [For a discussion of this technology, see, e.g., J. Cohen, Science, 259:1691-1692 (March 19, 1993); E. Fynan et al, Proc. Natl. Acad. Sci., 90: 11478-11482 (Dec. 1993); J. A. Wolff et al, Biotechniques, 11:474-485 (1991), all incorporated by reference herein, which describe similar uses of 'naked DNA']. For example, HCMV DNA encoding the IE protein under control of the HCMV-IE promoter may be used as an HCMV composition and administered according to the method of this invention. Other suitable homologous or heterologous promoter sequences may readily be selected, and the sequence constructed by methods known in the art.

Alternatively, the nucleic acid sequence composition may contain a vector which carries the CMV protein-encoding DNA under the control of regulatory sequences which are capable of directing the expression of the product of the sequence. Such vectors may be viral in origin. For example, a proposed HCMV vaccine using a recombinant vaccinia virus expressing HCMV glycoprotein B has been described. [See, e.g., Cranage, M. P. et al, EMBO J., 5:3057-3063 (1986).] Additionally, an adenovirus vector carrying an HCMV protein sequence or fragment has been described. [See, e.g., International Patent Application No. WO94/17810, published August 18, 1994 and International Patent

Application No. WO94/23744, published October 27, 1994]. Other viral vectors, such as canarypox virus or a retrovirus may also be employed as carriers for the nucleic acid sequences of HCMV proteins or fragments.

Similarly, such vectors may be non-viral in origin, such as known bacterial-based plasmids, e.g., pBR322 or pUC, or mammalian-based plasmids and may be employed to deliver the HCMV sequences to the subject. Many suitable plasmids are known to those of skill in the art and may be designed to contain the selected HCMV protein-encoding sequences.

As stated above for the protein compositions, the desired HCMV protein sequences or DNA sequences encoding them preferably are those HCMV proteins which readily induce CMI. Other HCMV proteins which may be desirable for use in this invention may induce low or undetectable amounts of antibody in the subject. It is presently preferred to use the HCMV protein sequences such as pp65, IE, and fragments thereof, such as IE exon 4.

The compositions of this invention may also employ more than one HCMV protein or fragment, or proteins and fragments from more than one strain of HCMV, or the nucleic acid sequences encoding same.

B. C. pneumoniae-containing Compositions

The compositions useful according to this invention are anti-microbial compositions directed against a *C. pneumoniae* infection.

In one embodiment, such compositions may be conventional antibiotics effective against many pneumonias, such as tetracycline, erythromycin and other conventional pharmaceutical antibiotic agents known to the skilled artisan.

In another embodiment an anti-microbial composition useful in this invention may contain an

immunogenic *C. pneunoniae* protein or fragment, e.g., the major outer membrane protein. Thus, the composition can be a killed *C. pneumoniae* of any suitable strain. Vaccines based on killed strains of *C. pneumoniae* may be prepared by conventional techniques of heat or irradiation.

Alternatively, the composition may contain only certain *C. pneumoniae* proteins, e.g., the major outer membrane protein, or fragments of these proteins which are characterized by the capacity to induce cell mediated or humoral immunity to *C. pneumoniae* infection upon administration of the protein in combination with an appropriate adjuvant. This composition may induce a low to undetectable anti-*C. pneumoniae* antibody response in the subject. A desired *C. pneumoniae* protein or peptides for this use is desirably a bacterial cell surface protein, which may be randomly fragmented. Random fragments of a *C. pneumoniae* protein may be readily tested for immunogenicity by one of skill in the art, using a variety of assay formats. Vaccines containing *C. pneumoniae* cellular antigens or fragments may be obtained conventionally, e.g., by cell lysis and standard purification or separation techniques.

Where the *C. pneumoniae* composition contains nucleic acid sequence(s) encoding a surface protein or fragment thereof, the nucleic acid sequence may be that of the selected protein itself, that is, a 'naked' DNA composition. So-called 'naked DNA' may be used to express the *C. pneumoniae* protein or peptide fragment *in vivo* under the control of suitable promoter sequences. [See the references cited above discussing naked DNA].

Alternatively, the nucleic acid sequence composition may contain a vector which carries the *C. pneumoniae* protein-encoding DNA under the control of regulatory sequences which are capable of directing the

expression of the product of the sequence. One particularly desirable embodiment is a vector encoding the major outer membrane protein. Suitable vectors may be selected from among known viral and plasmid vectors as described above for the HCMV compositions.

As stated above for the protein compositions, the desired *C. pneumoniae* protein sequences or DNA sequences encoding them preferably are those *C. pneumoniae* proteins which readily induce CMI. Other *C. pneumoniae* proteins which may be desirable for use in this invention may induce low or undetectable amounts of antibody in the subject.

The compositions of this invention may also employ more than one *C. pneumoniae* protein or fragment, or proteins and fragments from more than one strain of *C. pneumoniae*, or the nucleic acid sequences encoding same.

C. Combination Compositions

The present invention also provides for compositions which comprise both an anti-microbial agent directed against a *C. pneumoniae* infection and an anti-viral composition directed against HCMV in a suitable pharmaceutical carrier.

For example, a combination composition of the present invention may contain an HCMV protein containing composition such as described above and a *C. pneumoniae* protein composition as described above. Alternatively, a naked DNA composition may contain the DNA of an HCMV protein or fragment and a *C. pneumoniae* protein or fragment as described above. Similarly a composition may contain a mixture of plasmid or viral vectors individually bearing DNA sequences which encode an HCMV protein or fragment and a *C. pneumoniae* protein or fragment, as described above.

Another alternative combination composition may comprise a polycistronic vector, which carries the DNA

sequence which encodes an HCMV protein or fragment and the DNA sequence which encodes a *C. pneumoniae* protein or fragment. These fragments may be under the control of the same regulatory sequence which directs expression of both proteins *in vivo*. Alternatively, the polycistronic vector may contain separate regulatory sequences for each protein to be expressed.

Another embodiment of the composition of the present invention comprises the use of a conventional pharmaceutical antibiotic active against *C. pneumoniae* combined with an HCMV protein/DNA compositions as described above in a suitable pharmaceutical carrier.

A similar embodiment of the composition of the present invention comprises the use of a conventional pharmaceutical antiviral useful against HCMV infection combined with a *C. pneumoniae* protein/DNA composition as described above in a suitable pharmaceutical carrier.

II. Production of the Compositions

HCMV isolates, *C. pneumoniae* strains, proteins and fragments of these microorganism, and their DNA sequences may be obtained in a variety of conventional ways. For example, strains of HCMV useful in the practice of this invention may be obtained from depositories, such as the American Type Culture Collection, Rockville, Maryland (ATCC) or from other institutes or universities. Similarly, strains of *C. pneumoniae*, e.g., the TWAR strain, are available from the ATCC under Accession Nos. VR1310, 1355, 1356, 1360 and 2282, among others. Other strains may also be obtained from institutes or universities.

The preparation of heat-inactivated, or live attenuated viral and bacterial preparations are known to the art as provided by the publications incorporated above. Alternatively, protein sequences may be isolated

by conventional techniques from the many available strains. The sequences of the subunits of two HCMV strains have been published [See, e.g., Mach et al, J. Gen. Virol., 67:1461-1467 (1986); Cranage et al, (1986) cited above; and Spaete et al, Virol., 167:207-225 (1987)]. These sequences, and other known HCMV sequences, can be chemically synthesized by conventional methods known to one of skill in the art, [Sambrook et al, "Molecular Cloning", 2nd ed., Cold Spring Harbor, NY (1989)] or the sequences purchased from commercial sources.

Similarly fragments of *C. pneumoniae* surface proteins may be randomly fragmented by conventional means, e.g., restriction enzymes, and sequenced and chemically synthesized by employing the same protocols.

In the practice of another embodiment of this invention the HCMV protein or fragment and/or the *C. pneumoniae* immunogenic protein or fragment may be produced *in vitro* by recombinant techniques in large quantities sufficient for use in a composition of this invention. Alternatively, a recombinant virus or plasmid vector carrying the HCMV protein or fragment and/or *C. pneumoniae* protein or fragment may be prepared by conventional techniques of genetic engineering. See, for example, the techniques described in the above-cited international patent applications, incorporated by reference herein.

The preparation of a pharmaceutically acceptable composition containing the HCMV and/or *C. pneumoniae* protein(s) or DNA sequence(s), having appropriate pH, isotonicity, stability and other conventional characteristics is within the skill of the art. These pharmaceutical compositions may optionally contain other components, such as adjuvants, e.g., aqueous suspensions

of aluminum and magnesium hydroxides, and/or pharmaceutically acceptable carriers, such as saline.

It is anticipated that CMV other than HCMV, and various strains of *C. pneumoniae* may be used to design compositions useful to prevent analogous conditions in various animal species, i.e., domestic animals and other valuable animals.

III. Methods of the Invention

Without wishing to be bound by theory, and as described in more detail herein, the inventors theorize, and their preliminary results suggest, that AT is a multifactorial process, with CMV and/or *C. pneumoniae* infections initiating development of the disease. Reactivation of these pathogens as well as genetic factors related to lipid metabolism contribute to the disease. CMV is involved in two of the major mechanisms that lead to the development of AT. The first is efficient infection of cells in the inner layer of arteries *in vivo* and induction of and injury at the area of infection corresponding to early AT lesions. The second mechanism is an increase in serum levels of low-density lipoprotein cholesterol, a major lipid contributor to AT plaques. It is anticipated that chronic infection with periodic reactivations, typical of human CMV infections, leads to full-blown AT. Additionally, seroepidemiologic data and direct isolation of *Chlamydia pneumoniae* from human AT plaques also suggest the involvement of this bacterium in the development of AT. Coinfection or consecutive infection of the arterial wall with CMV and *C. pneumoniae* likely increase the cellular damage of the intima that leads to the development of mature AT plaques.

Thus, the invention provides preventive and therapeutic compositions and methods that reduce the

incidence of these closely related infections in certain subjects and hence those conditions and diseases characterized by injury to arterial vessels. Suitable subjects for treatment with the compositions and methods of the present invention include mammals, preferably humans, (a) who are anticipating immunosuppression due to organ or bone marrow transplant (for administration before, during or after the transplant); (b) pre-atherosclerotic subjects seeking prophylaxis of atherosclerosis, e.g., human children or infants; (c) subjects with existing atherosclerosis, e.g., human adults; (d) subjects who have restenosis, an accelerated form of AT, which is the narrowing of coronary arteries that occurs in about half of patients after coronary atherectomy and balloon angioplasty; and (e) subjects seeking to prevent or ameliorate the occurrence of restenosis, i.e., patients anticipating coronary atherectomy or balloon angioplasty, or post-surgical patients, among others. It should be understood that any injury to an arterial vessel, either existing or anticipated, can benefit by the use of the present invention.

According to one embodiment of this invention, a subject is administered an effective amount of a composition which contains either (a) a human cytomegalovirus (HCMV) protein or fragment thereof, or (b) a nucleic acid sequence encoding HCMV protein or fragment thereof. In either case, the composition is administered in an amount sufficient to induce CMI and humoral immunity to CMV infection and prevent, or retard, the development of atherosclerotic lesions.

According to another embodiment of this invention, a subject is administered an effective amount of a composition which contains either (a) a *C. pneumoniae* protein or fragment thereof, or (b) a nucleic

acid sequence encoding said protein or fragment thereof. In either case, the composition is administered in an amount sufficient to induce CMI and humoral immunity to *C. pneumoniae* infection and prevent, or retard, the development of atherosclerotic lesions.

According to yet another embodiment of this invention, a subject is administered an effective amount of a composition which contains a combination of (a) a *C. pneumoniae* protein or fragment thereof and a human cytomegalovirus (HCMV) protein or fragment thereof, or (b) a nucleic acid sequence encoding said *C. pneumoniae* protein or fragment thereof and a nucleic acid sequence encoding HCMV protein or fragment thereof. In either case, the composition is administered in an amount sufficient to induce CMI and humoral immunity to both *C. pneumoniae* and HCMV infection and prevent, or retard, the development of atherosclerotic lesions due to either infective agent.

Still another embodiment of this invention is a treatment method by which a subject as described above is administered an effective amount of an anti-microbial composition effective in treating *C. pneumoniae* infection. Such a method may further include contemporaneous treatment (i.e., before, during or after administration of the anti-microbial agent) with a composition containing either a cytomegalovirus (HCMV) protein or fragment thereof, or a nucleic acid sequence encoding said HCMV protein or fragment thereof, the HCMV-containing composition administered in an amount sufficient to induce CMI and humoral immunity to HCMV infection. Such a method enables the prevention or retardation of the development of atherosclerotic lesions due to either infective agent.

Still another novel treatment or prophylactic method of this invention involves the use of a

composition which contains a combination of an immunogenic *C. pneumoniae* protein or fragment thereof and an antiviral composition directed against human cytomegalovirus. Another embodiment of this method may also employ a nucleic acid sequence encoding the *C. pneumoniae* protein or fragment thereof. In either case, the *C. pneumoniae*-containing composition is administered in an amount sufficient to induce CMI and humoral immunity to *C. pneumoniae*. This method enables the prevention or retardation of the development of atherosclerotic lesions due to either infective agent.

According to the present invention, the selected composition described above is administered preferably as a vaccine to infants. Subsequent periodic boosters may be administered as for other vaccines.

Alternatively, the method involves administering the compositions of the present invention to patients prior to, or immediately after, undergoing organ or bone marrow transplants, blood transfusions, or other immunosuppressive treatments. Organ transplant patients can develop atherosclerosis very quickly in a transplanted organ, either through the organ itself or through blood transfusions. Thus, the method of this invention is useful for treating organ transplant, or otherwise, immunosuppressed patients, following the transplantation.

Still another alternative method of the invention involves administering one of more of the described compositions to a balloon angioplasty or coronary atherectomy patient, either before, during or after the surgical procedure to prevent or reduce the development of restenosis.

It is anticipated that therapeutic treatments of compositions described above to adult subjects having some existing degree of atherosclerosis is also useful to

slow the advance of the condition and/or prevent or reduce further injury, i.e., the occurrence of atherosclerotic injury to blood vessels.

Another clinical setting for which the methods of the present invention are useful is for a woman planning pregnancy. To avoid passage of CMV to a fetus, which, when it occurs, causes congenital malformations and heart complications, a woman planning a pregnancy may be treated with a CMV composition according to this invention.

For the compositions containing proteins, the preferred dosage ranges from about 10 to about 80 micrograms of protein. In a combination composition, the dosages of each protein are at the lower end of that range. Preferred dosages, in general, are the lower end of that range. When a recombinant virus vector is administered as the pharmaceutical composition, a dosage of between 10^5 and 10^7 plaque forming units of each vector may be used. Preferred dosages are the lower end of that range. When naked DNA is administered as the pharmaceutical composition, a dosage of between 50-200 micrograms may be used. Preferred dosages are the lower end of that range. Dosages of conventional antibiotics are administered by conventional routes and dosages ordinarily prescribed for such pharmaceutical compositions and these may also be determined by the attending physician.

Additional similar, repeated doses of the protein/nucleic acid sequence-containing compositions of this invention may also be administered at a desired interval, i.e., at 1-10 year intervals, where considered desirable by the physician. The dosage regimen involved in the method for treating or preventing atherosclerosis with such compositions can be determined considering

various clinical and environmental factors known to affect such administration.

The route of administration may be oral, nasal or subcutaneous, particularly where the virus vector composition is used. However, another route of administration may include intramuscular injection. The route of administration depends upon the particular composition selected for such use, and may readily be selected by the attending physician.

The following examples illustrate various aspects of this invention and do not limit the invention, the scope of which is embodied in the appended claims. BALB/c and C57BL/6 mice were used in these experiments.

Example 1 - Development of Early Atherosclerotic Lesions in Mice Infected with Murine CMV

A. Infection

Mice were infected with murine CMV (Smith strain; ATCC VR-194) at a dose of $1X10^6$ pfu via the intraperitoneal (i.p.) route on day 1. Fifty-five days post infection, the mice were sacrificed and the heart was obtained and sectioned above the right and left auricles. These tissues were subjected to a conventional immunofluorescence assay using anti-MCMV polyclonal antibody.

Endothelial cells (EC) and smooth muscle cells (SMC) of the mouse aortic wall express viral antigens after MCMV infection. Accumulation of inflammatory and SMCs in the aortic lumen at the site of viral antigen expression has been observed and defined as early atherosclerotic lesions.

B. Additional Experiments

In the next series of experiments, mice were infected i.p. with the parental strain of MCMV at a dose of 1×10^4 pfu, γ-irradiated at the time of infection

(day 0) or non-irradiated, and fed an atherogenic or normal diet. Mice were sacrificed on day 36, hearts were obtained and sectioned for viral antigen expression and pathological changes.

Figs. 1A-1C show sections of a naive mouse, with no reactivity with the anti-MCMV antibody in the immunofluorescence (IF) test and with no inflammatory reaction in the aortic wall and lumen. Figs. 1G-1I show large areas of virus antigen expression with adherent inflammatory cells on the luminal surface of the aorta from a mouse infected, immunosuppressed and fed a normal diet. The inflammatory infiltrate consists of many large lymphocytes and plasma cells with a few monocytes and peripheral mononuclear cells. Similar expression of viral antigens and inflammatory reactions was detected in the aortic wall of a mouse infected, immunosuppressed, and fed an atherogenic diet. Small areas of the aortic wall with expression of MCMV antigens and accumulation of inflammatory cells at a site of viral antigen expression in a mouse infected, not immunosuppressed, and fed cholesterol or normal diet were also observed. Mice not infected but fed a cholesterol diet, or not infected but irradiated, showed neither viral antigen expression nor inflammatory changes in the aorta.

These data show that an acute MCMV infection of mice induces a vascular wall inflammation that may play a role in the immune injury of the vascular structure and thus initiate the development of AT.

C. Immunosuppression

Mice were infected with murine CMV (Smith strain) by the same procedure as described above. The mice were exposed to gamma-irradiation (500 rad) at the time of infection. Thirty-six days post infection, the mice were sacrificed and various tissues were examined as described above.

Immunosuppression by gamma-irradiation at the time of virus infection increased the virus titer in salivary glands from $1-5x10^3$ to $0.5-2.5x10^4$ pfu/salivary glands and extended the size of the expression of viral antigens in the aortic wall from about 2/10 of aortic wall (Fig. 1J) to 5/10 (Fig. 1G) or 7/10 (Fig. 1D). Accumulation of inflammatory cells in the lumen and in the periaortic area was seen at the region where viral antigen expression was detected in mice not irradiated (Figs. 1J-1L). More irradiated inflammatory cells were seen throughout the aortic lumen.

D. High Cholesterol Diet

Another group of mice was fed a high cholesterol Western diet designed to elevate plasma cholesterol levels for 1 week, then infected with MCMV as described in the assays above. Fifty-five days post infection, the mice were sacrificed and vascular tissues were examined as described above.

The high cholesterol diet had no significant synergistic effect on the development of early atherosclerotic lesions at the site of viral antigens.

E. Serum Cholesterol - *in vivo* assay

Three groups of mice were treated as follows: One group was fed with a high cholesterol diet and infected with MCMV; a second group was fed with a normal diet and infected with MCMV, and a third group was not infected and used as a control. After fifty-five days post infection (or on the analogous day for the controls), serum from each group of mice was collected and assayed for total cholesterol and triglycerides using a commercially available (Sigma) kit.

Analysis of the sera of each group demonstrated that the serum level of low density cholesterol (LDC), a major lipid component of lipid-leded macrophages and other cells in the atherosclerotic plaques, is

significantly increased in mice infected with MCMV, either fed with atherosclerotic diet (as in Part C above) or not, as compared with uninfected mice.

F. Lipid Metabolism - *in vitro* assay

A human arterial smooth muscle cell line (cells No. 101 obtained from Dr. Elliot Levine, The Wistar Institute, Philadelphia, PA) was infected *in vitro* with human CMV at a multiplicity of infection (MOI) of 1-2. After five days, the cells were extracted with chloroform-methanol 2:1 and lipid extract analyzed for total and free cholesterol using standard methodology.

In the infected cells, the esterified cholesterol component of the total cholesterol was 29.7%, as compared with the undetectable level of esterified cholesterol in uninfected cells. These results, and those of part D above, indicate that both HCMV and MCMV disturb lipid metabolism in infected cells and change it in the direction of the accumulation of lipid elements, contributing to the development of atherosclerotic plaques.

Example 2 - Treating Mice with CMV Compositions

Tissue culture-adapted (attenuated) MCMV (ATCC No. VR-104) was grown in tissue culture fibroblasts prepared from BALB/c embryos by conventional techniques. Alternatively, the tissue culture-adapted MCMV was inactivated by treatment with heat at 56°C for 30 minutes.

BALB/c mice (10 in each group) were immunized i.p. with either the live attenuated MCMV at a dose of $1x10^6$ pfu in a volume of 0.1 ml or the inactivated virus (0.1 ml) on day 0. Mice received a booster inoculation three weeks after the first inoculation. A group of mice was not immunized for use as controls.

Five weeks after the second inoculation, all mice were challenged with $3x10^6$ pfu live virulent MCMV, which was grown *in vivo* in murine salivary glands. On day 10 after the challenge, the mice were sacrificed and their vascular tissue examined by immunofluorescence (IF) assay using an anti-murine cytomegalovirus (MCMV) polyclonal mouse serum.

The control mice were found to have viral antigen expression in the aortic wall and in smooth muscle cells of the heart, and early atherosclerotic lesions. However, mice immunized with either live attenuated, or heat inactivated, attenuated MCMV were found to have no such lesions. Thus, the live or inactivated attenuated MCMV compositions protected the mice from the development of viral antigen expression and early atherosclerotic lesions following live virulent viral infection, indicating that the development of atherosclerosis is preventable.

Example 3 - Mice Infected with HCMV

This example demonstrates the effect on the development of early atherosclerotic lesions in the mouse aortic intima of infection of mice with HCMV.

HCMV does not replicate in mouse cells, but expresses immediate early (IE) antigens. The expression of CMV IE antigens may be sufficient to cause such lesions. This theory is tested by examining attachment of inflammatory cells and SMCs to the intima, expression of IE antigens and cellular adhesion molecules in the ECs and SMCs.

BALB/c mice are injected i.v. with live purified HCMV at a dose of 10^4 - 10^6 pfu. Two to twenty days after infection, mice are sacrificed, heart sections obtained as described above, and expression of IE antigens is tested by IF assay using a monoclonal

antibody specific to IE antigens (e.g., E13, Chemicon). The presence of early atherosclerotic lesions is analyzed on consecutive sections stained with hematoxilin-eosin. The presence of significant lesions indicates that IE expression alone can cause early atherosclerotic damage.

Example 4 - Latency Murine CMV Model of HCMV Infection

This experiment describes a latency CMV murine model which mimics the course of HCMV infection in humans:

Mice are inoculated with MCMV, thereby establishing an acute infection, and tissue is tested for atherosclerotic lesions and expression of CMV viral antigens as described above. After the virus becomes latent, i.e., 3-4 weeks, the same tests are performed and viral antigens are not expressed.

Mice are then immunosuppressed, preferably by gamma irradiation to reactivate the CMV infection. The same tests as described above are performed and viral antigens are again expressed. After the virus again becomes latent, the tissues are examined and viral antigens are no longer expressed.

A periodic increase in the severity of atherosclerotic lesions accompanies the periodicity of virus reactivation.

A second set of mice are similarly treated as above, but immunized with the selected CMV composition either before acute infection or in the latency stage. Comparison of atherosclerotic lesions in control mice vs. immunized mice at the various stages of the model demonstrates the efficacy of immunizing the mice with a CMV composition of this invention to prevent the development or further progression of such lesions.

Example 5 - Rabbit Model of HCMV

The rabbit is a well-established model for experimental atherosclerosis. Rabbits are infected with HCMV or MCMV and the aortic tissue tested as above described for expression of IE antigens, as well as for the development of fatty streaks, i.e., the early lesions of atherosclerosis (AT). Since the rabbit aorta is considerably larger than the mouse aorta, the fatty streaks are visible macroscopically.

Once AT lesions develop in the rabbit aorta after CMV infection, the CMV compositions described herein are tested for the ability to protect the animal against further progression of AT lesions.

Example 6 - Development of Early Atherosclerotic Plaques and Antigens

A. BALB/c and C57BL/6 mice were infected i.p. with either 1 x 10^4 pfu a recombinant MCMV expressing the bacterial ß-galactosidase (lacZ) gene (MCMV-lacZ) [C. A. Stoddart *et al*, J. Virol., 68:6243-6253 (1994) (gift of Dr. E. Mocarski, Stanford University, Stanford, CA), or with the same dosage of the parental MCMV (Smith strain, ATCC VR-194). BALB/c mice were infected i.p. with 1 x 10^4 pfu of MCMV-lacZ virus, and immunosuppressed by γ-irradiation (450 rad) at the time of infection to slow down virus clearance, and fed either an atherogenic diet (8% coconut oil, 2% soybean oil, 0.5% cholesterol) or a normal mouse diet. Uninfected mice were treated similarly. Mice were sacrificed 25 days after virus infection, and hearts were obtained and sectioned as described in B. Paigen *et al*, Atheroscl., 68:1614-1620 (1987). Sections were treated with X-gal to detect lacZ-expressing cells indicating virus replication, or stained with H&E for pathological changes or with oil red O for lipid accumulation in the tissues.

An inflammatory response developed in the aortic wall of the mice, similar to early AT lesions in humans, and lipid metabolism shifted to high levels of serum LDL-C, a major contributor to AT plaques. MCMV-lacZ replicated and induced inflammatory reactions at the site of replication in the aortic wall (Fig. 2A).

In mice infected with MCMV-lacZ, irradiated and fed an atherogenic diet, lacZ was expressed in the endothelial and smooth muscle cells of the aorta (Figs. 2A-2C). An early AT plaque at the site of virus replication with inflammatory cells in the lesion and below it through the thickness of the aorta was also seen (Fig. 2A-2C). Oil red O staining revealed lipid in this same area (not shown). Mice infected with the virus, irradiated and fed a normal mouse diet also showed lacZ-expression in the salivary gland and arterial adventitia associated with adventitial vacuoles, in a few muscle cells in the heart (not shown). Mice infected with the virus, irradiated and fed a normal mouse diet also showed lacZ-expression in the salivary gland and arterial adventitia associated with adventitial vacuoles, in a few muscle cells in the heart (not shown), as well as in the subendothelial space of a pulmonary vein (not shown). Uninfected and irradiated mice fed either an atherogenic or a normal diet showed no pathological changes.

These results provide the first evidence that acute MCMV infection induces an inflammatory response in the aortic wall of mice, resembling early AT lesions found in humans.

Example 7 - Influence of MCMV on Lipid Metabolism

To test how MCMV infection influences the lipid metabolism of mice fed an atherogenic or normal diet, sera were obtained from mice, and total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C)

determined. Results showed that TC levels in mice infected but fed normal diet were very similar to those in the naive mice, while TC levels were higher in mice fed the cholesterol diet, either infected or uninfected.

The results also showed that MCMV-infected normocholesterolemic mice had a significantly higher percentage of LDL-C in the serum than naive mice. Similarly, significantly higher LDL-C levels were found in mice fed an atherogenic diet and infected than in mice fed the same diet but uninfected. The results indicate that MCMV infection in mice disturbs lipid metabolism and shifts it to higher levels of LDL-C.

Example 8 - Effects of *C. pneumoniae* Infection

BALB/c and C57BL/6 mice were infected intranasally with *C. pneumoniae* (10^7 $TCID_{50}$) and sacrificed on days 3, 7, 11, and 27. Hearts were obtained and sectioned for *C. pneumoniae* antigen expression and pathological changes. Fig. 3A shows expression of *C. pneumoniae* antigens in the aortic wall and Fig. 3B shows an inflammatory reaction at the site of *C. pneumoniae* replication.

Human arterial smooth muscle cells (HASMC, obtained from Dr. E. Levine, The Wistar Institute) infected with human CMV (HCMV) or *C. pneumoniae* expressed HCMV antigens (Fig. 4A) or *C. pneumoniae* antigens (Fig. 4B), indicating the replication of each pathogen in these cells.

Example 9 - The causative role of acute and reactivated CMV infection in the initiation and development of early and mature AT plaques

The following experiment is conducted to establish to what extent latent and reactivated MCMV infection, alone or in combination with an atherosclerotic diet, increases the number and severity of AT plaques in mice,

and to analyze the role of CMV and contribution of hypercholesterolemia to the development of AT initiated by CMV.

A. Sixteen groups (see Table 1) of C57BL/6 normal female mice (even-number and odd-number groups of 30 and 40 mice, respectively, 8-10 weeks of age, Charles Rivers Laboratory) with no genetic disorders are infected i.p. with MCMV grown previously in salivary glands, at a dose 1×10^4 pfu/mouse. A group of these mice are immunosuppressed by γ-irradiation (450 rads) at the time of infection and a group of these mice are not irradiated. Each group is then fed an atherogenic or a normal diet throughout the experiment.

Mice in even-number groups are further irradiated (450 rads) 2 times during the observation period of 300 days to reactivate latent CMV infection. The *in vivo* mouse model for the acute (infectious virus detectable), latent infectious virus no longer detectable), and reactivated (infectious virus detectable again) phases of MCMV infection in the salivary glands, lungs, spleens, and kidneys has already been developed.

Ten mice of odd-number groups (acute infection) are sacrificed on days 25, 50, 225, and 300, while 10 mice of even-number groups (latent or reactivated infection) are sacrificed on days 125, 225, and 300. Hearts are then processed: (i) for the qualitative and quantitative evaluation of AT plaques and for the determination of lipid accumulation by staining sections of aorta with H&E and oil red O; (ii) for the presence of MCMV antigens in sections by immunofluorescence assay using hyperimmune serum from MCMV-infected mice; (iii) for the presence of viral mRNA and DNA during acute, latent, and reactivated stages of infection using RNA and DNA extracted from the upper part of the hearts (not sectioned) for RT-PCR and PCR analysis with appropriate

primers for the immediate early or late genes; (iv) for the expression of mRNA and/or proteins of the Hsp family, and growth factors, cytokines, adhesion molecules, e.g., vascular endothelial growth factor, Le^x, ICAM-1, VCAM-1, NO, IL-12, IL-8, IL-10, and IFN-γ.

Mouse sera are tested for the percentage of LDL-C by determining TC and HDL-C, as well as for serum ICAM-1 and serum VCAM-1 by ELISA.

Analysis of the aorta obtained from genetically normal mice reveals the extent to which acute MCMV infection initiates the development of early AT plaques; whether a latent MCMV infection with periodic reactivations, a common characteristic of HCMV infections in humans, furthers early plaques into mature, complicated plaques. The influence of a cholesterol diet applied throughout the experiment is evaluated, and immunosuppression applied at the time of infection to prolong initial virus replication, on the number and severity of AT plaques in the aorta.

Finally, the extent to which MCMV immediate early and late gene DNA and mRNA are present in the acute, latent, and reactivated stages of infection in the aortic wall of the mice is observed and the cellular mechanisms involved in the development of AT.

Analysis of mouse sera provides further information about the pathomechanisms of this complex disease, and is predicted to reveal that acute and chronic MCMV infection shifts lipid metabolism to a high percentage of LDL-C in genetically normal mice, as suggested by the preliminary experiments.

B. ApoE-deficient and/or LDL receptor-deficient mice (Jackson Laboratories) are infected or uninfected, and treated as described for normal mice in odd-number groups in Table 1. Each group consists of 10 mice, of which 5 are sacrificed on day 25 and 5 are sacrificed on

day 50 after initial infection and treatment. Hearts and sera are obtained and examined as described for genetically normal mice.

The qualitative and quantitative analysis of AT plaques, as well as the kinetics of development, indicate how MCMV infection alters the number and complexity of these plaques in the genetically hypercholesterolemic mice, which show strong similarities to genetic deficiencies in humans. Since the genetically hypercholesterolemic mice develop mature AT plaques after MCMV infection, the ratio of apoptotic cells is determined by terminal-deoxynucleotidyl transferase mediated DNA-end labeling (TUNEL).

Preliminary results show that MCMV replicates in the aortic wall of BALB/c and C57/BL mice.

Table 1. Treatment protocol for normal mice

	Immediate treatment			Days of virus reactivation by γ-irradiation	
Groups	I*	D*	G*	100	200
1	+	--	--	--	--
2	+	--	--	+	+
3	+	--	+	--	--
4	+	--	+	+	+
5	+	+	--	--	--
6	+	+	--	+	+
7	+	+	+	--	--
8	+	+	+	+	+
9	--	--	+	--	--
10	--	--	+	+	+
11	--	+	--	--	--
12	--	+	--	+	+
13	--	+	+	--	--
14	--	+	+	+	+
15	--	--	--	--	--
16	--	--	--	+	+

*I - intraperitoneal infection with MCMV; D = cholesterol diet;
G = γ-irradiated

Example 10 - Contribution of coinfection with MCMV and *C. pneumoniae* to the development of AT lesions

Eleven groups of C57/BL6 mice (20/group) (Charles River Laboratory) are infected and treated as outlined in Table 2. Inoculation with the second pathogen is carried out on day 25 after inoculation with the first pathogen to avoid possible interference between the susceptibility of cells to these two pathogens, and to allow sufficient time for the development of an inflammatory response to the first pathogen.

Ten mice are sacrificed 25 days after inoculation with the second pathogen. As controls, mice infected with *C. pneumoniae* are tested with appropriate antibiotics against *C. pneumoniae* to establish *C. pneumoniae*-specificity of the lesions in mice infected with the bacterium, but not treated with antibiotics.

Hearts are processed for qualitative and quantitative evaluation of AT lesions; presence of MCMV and *C. pneumoniae* antigens in the aortic wall by immunofluorescence assay; presence of cellular Hsp 70 family, vascular endothelial growth factor, Le^x, ICAM-1, VCAM-1, NO, IL-12, IL-8, IL-10, and IFN-γ, and presence of apoptotic cells in the lesions by TUNEL.

Sera are processed for determination of TC and LDL-C levels; and serum ICAM-1, serum VCAM-1 levels and Hsp 70 antibodies. Lungs and livers are processed for infectious MCMV and *C.pneumoniae*.

These analyses show whether the injury initiated by MCMV infection progresses to accelerated and/or more complicated lesions as a result of *C. pneumoniae* superinfection, and whether primary *C. pneumoniae* infection initiates aortic wall injury that develops into AT upon secondary MCMV infection. Also, these analyses show whether MCMV or *C. pneumoniae*-induced apoptosis is involved in the development of mature plaques, and

whether cellular molecules are induced that are involved in the development of AT.

A decrease of susceptibility of aortic wall (or the whole mouse) to the second pathogen due to induction of some cellular factors (e.g. IFN-α) by the first pathogen is expected to be only transient. Scheduling the second infection at different times after the first infection overcomes this difficulty.

Table 2. Treatment protocol for MCMV- and C. pneumoniae-coinfected mice

	Immediate treatment		Later Treatment	
Groups	Infection	γ-irradiation	Infection	γ-irradiation
1	MCMV	+	C.pneumoniae	--
2	MCMV	+	-	--
3	--	+	C.pneumoniae	--
4	--	--	C.pneumoniae	--
5	--	+	-	--
6	C.pneumoniae	--	MCMV	+
7	C.pneumoniae	--	-	--
8	--	--	MCMV	+
9	--	--	MCMV	--
10	--	--	-	+
11	--	--	-	--

Example 11 - Coinfection of human arterial endothelial and smooth muscle cells in vitro with HCMV and C. pneumoniae influences their replication

Both HCMV and *C. pneumoniae* replicate in human arterial endothelial and smooth muscle cells. The interaction between the replication of these two pathogens and the effects of the coinfecting pathogens on the infected endothelial, smooth muscle, or any other cells are addressed as follows.

A. *Coinfection experiments*

The following cells cultures: (a) human aortic endothelial cells (obtained from Dr. Jay Nelson, Oregon

Health Sciences University, Portland, OR); (b) human iliac arterial endothelial cells; and (c) human iliac arterial smooth muscle cells (both obtained from Dr. Eliott Levine, The Wistar Institute), are treated as follows.

Cell cultures are infected with HCMV and *C. pneumoniae* simultaneously. Other cell cultures are infected consecutively (with HCMV followed by *C. pneumoniae*, with *C. pneumoniae* followed by HCMV. Still another set of cell cultures is infected with only one of these pathogens. Control cultures are uninfected.

Co-infected cultures are tested for replication kinetics of the two pathogens, by quantitating the expression of *C. pneumoniae* and CMV-immediate antigens (IE) in immunofluorescence tests using monoclonal antibodies specific to these antigens, and by quantitating the production of infectious CMV and *C. pneumoniae* in susceptible cell cultures. The quantitative expression of various adhesion molecules are tested by immunochemical staining. Production of various cellular factors, including the Hsp60 family, NO synthetase, and vascular endothelial growth factor are tested by immunological methods. The ratio of apoptotic cells in infected cultures is tested by sodium-iodide staining with subsequent flow-cytometric determination of the percentage of hypodiploid-nuclei.

For assessment of colocalization of apoptotic cells with infected cells, a double-staining process including TUNEL and pathogen-specific immunostaining is performed. Adhesion molecules, as well as Hsp60, are thought to be involved in the development of AT. The presence of apoptotic cells in human AT lesions has been demonstrated and MCMV is known to induce apoptosis of T cells.

These experiments reveal: (a) how these two pathogens influence the replication of each other; (b) how the expression of adhesion molecules and various cellular factors is influenced by these co-infecting pathogens, as compared with that of the single pathogen-infected cultures; and (c) how apoptosis is induced by the two pathogens in cells infected with one pathogen or with two pathways sequentially.

B. Transfection and infection

Because the human CMV-IE proteins, especially the IE2 protein, are potent transactivators of heterologous promoters, *C. pneumoniae* replication and the production of adhesion molecules and various growth factors in cell lines stably transfected with eukaryotic expression plasmids expressing the IE1 and 2 (pRL43a) or only the IE2 protein of HCMV (pMC18) [both obtained from Dr. Gary Hayward of Johns Hopkins University, Baltimore, MD)] are analyzed. A stably transfected rhabdomyoma cell line was transfected with these plasmids. The parental cell lines are tested for susceptibility to *C. pneumoniae* infection. Stably transfected Hep2 or McCoy cells, which are highly susceptible for *C. pneumoniae* are also transfected.

Transfected cultures are tested for replication of *C. pneumoniae* as well as for the expression of adhesion molecules and various cellular factors and mechanism(s) as described for the co-infection experiments.

All published documents are incorporated by reference herein. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. Use of a human cytomegalovirus (HCMV) protein or fragment thereof in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to the mammal in an amount inducing cell mediated immunity and/or antibody response to HCMV in the mammal.

2. Use according to claim 1 wherein said HCMV protein is selected from the group consisting of HCMV proteins pp65, IE, IE-exon-4, pp150, gB, gH, pp28, and pp52.

3. Use according to claim 1 or 2 wherein the medicament is administered to a mammal in a low dose comprising between about 10 and about 80 mgs protein per inoculation.

4. Use of a nucleic acid sequence encoding a human cytomegalovirus (HCMV) protein or fragment thereof in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to a mammal in an amount inducing cell mediated immunity and/or antibody response to HCMV in the mammal.

5. Use according to claim 4 wherein said medicament comprises an attenuated, live HCMV.

6. Use according to claim 4 wherein said medicament comprises an inactivated HCMV.

7. Use according to claim 4 wherein said nucleic acid sequence is a recombinant viral vector comprising a nucleic acid sequence encoding said protein or fragment under the control of a regulatory sequence capable of directing expression of said HCMV protein sequence.

8. Use according to claim 7 wherein the recombinant viral vector is a recombinant virus selected from the group consisting of adenovirus, poxvirus, and retrovirus.

9. Use according to any of claims 4 to 8 wherein said medicament is administered in a low dose comprising of between 10^3 and 10^7 pfu per inoculation.

10. Use according to any of claims 1 to 9, characterized in that the medicament is co-administered to said mammal with an immunogenic *Chlamydia pneumoniae* protein or fragment thereof.

11. Use according to any of claims 1 to 9, characterized in that the medicament is co-administered to said mammal with a nucleic acid sequence encoding an immunogenic *C. pneumoniae* protein or fragment thereof.

12. Use according to claim 11, wherein the immunogenic *C. pneumoniae* is administered in a low dose comprising between 50 and 200 μg DNA per inoculation.

13. Use according to any of claims 1 to 9, characterized in that the medicament is co-administered to said mammal with an effective amount of an anti-microbial agent effective against *C. pneumoniae* infection.

14. Use according to claim 13 wherein said anti-microbial agent comprises a chemical composition which kills *C. pneumoniae in vivo*.

15. Use according to any of claims 10 to 14, wherein said co-administering step occurs before, during or after said administering of said medicament.

16. Use of an immunogenic *Chlamydia pneumoniae* protein or fragment thereof in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to a mammal in an amount which induces cell mediated immunity and/or antibody response in the mammal.

17. Use according to claim 16 wherein said composition is a killed *C. pneumoniae*.

18. Use according to claim 16 wherein said amount is a low dose comprising between about 10 and about 80 mgs protein per inoculation.

19. Use of a nucleic acid sequence encoding an immunogenic *C. pneumoniae* protein or fragment thereof in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein the medicament is administered to the mammal in an amount which induces cell mediated immunity and/or an antibody response upon expression of said protein or fragment in said mammal.

20. Use according to claim 19 wherein said medicament comprises a recombinant vector comprising a nucleic acid sequence encoding said protein or fragment under the control of a regulatory sequence capable of directing expression of said *C. pneumoniae* protein sequence.

21. Use according to claim 20 wherein said vector is a virus selected from the group consisting of adenovirus, poxvirus, and retrovirus.

22. Use according to any of claim 19 to 21 wherein said amount is a low dose comprising of between 50 and 200 μg of DNA per inoculation.

23. Use of an anti-microbial composition in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein the medicament is administered in an amount effective to reduce or eliminate *C. pneumoniae* infection.

24. Use according to claim 24 wherein said anti-microbial agent comprises a chemical composition which kills *C. pneumoniae in vivo*.

25. A composition useful for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal comprising, in a suitable pharmaceutical carrier,:

(a) an amount of an anti-microbial composition effective to reduce or eliminate *C. pneumoniae* infection; and

(b) an amount of an anti-viral composition effective to reduce or eliminate HCMV infection.

26. The composition according to claim 25 wherein said anti-microbial composition (a) is selected from the group consisting of:

(i) a chemical composition which kills *C. pneumoniae in vivo*;

(ii) a composition comprising an immunogenic *Chlamydia pneumoniae* protein or fragment thereof, said amount inducing cell mediated immunity or cell mediated immunity and antibody response directed against said *C. pneumoniae* in a mammal; and

(iii) a nucleic acid sequence encoding an immunogenic *C. pneumoniae* protein or fragment thereof, said composition inducing cell mediated immunity and/or an antibody response directed against *C. pneumoniae* upon expression of said protein in a mammal.

27. The composition according to claim 25 or claim 26 wherein said composition (b) is selected from the group consisting of:

(i) an anti-viral chemical reagent;

(ii) a composition comprising an HCMV protein or fragment thereof, said amount inducing cell mediated immunity or cell mediated immunity and antibody response directed against said HCMV in a mammal; and

(iii) a nucleic acid sequence encoding an HCMV protein or fragment thereof, said composition inducing cell mediated immunity and/or an antibody response directed against HCMV upon expression of said protein in a mammal.

28. Use of a composition according to any of claims 25 to 27 in the preparation of a medicament for the treatment of atherosclerotic lesions or restenosis.

AMENDED CLAIMS

[received by the International Bureau on 12 January 1998 (12.01.98); original claim 1 amended; original claims 23 and 24 cancelled; new claim 29 added; remaining claims unchanged (3 pages)]

1. Use of a human cytomegalovirus (HCMV) protein in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to the mammal in an amount inducing cell mediated immunity and/or antibody response to HCMV in the mammal

2 Use according to claim 1 wherein said HCMV protein is selected from the group consisting of HCMV proteins pp65, IE, IE-exon-4, pp150, gB, gH, pp28, and pp52

3 Use according to claim 1 or 2 wherein the medicament is administered to a mammal in a low dose comprising between about 10 and about 80 mgs protein per inoculation

4 Use of a nucleic acid sequence encoding a human cytomegalovirus (HCMV) protein or fragment thereof in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to a mammal in an amount inducing cell mediated immunity and/or antibody response to HCMV in the mammal

5. Use according to claim 4 wherein said medicament comprises an attenuated, live HCMV

6 Use according to claim 4 wherein said medicament comprises an inactivated HCMV

20 Use according to claim 19 wherein said medicament comprises a recombinant vector comprising a nucleic acid sequence encoding said protein or fragment under the control of a regulatory sequence capable of directing expression of said C *pneumoniae* protein sequence

21. Use according to claim 20 wherein said vector is a virus selected from the group consisting of adenovirus, poxvirus, and retrovirus

22 Use according to any of claim 19 to 21 wherein said amount is a low dose comprising of between 50 and 200 μg of DNA per inoculation

25 A composition useful for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal comprising, in a suitable pharmaceutical carrier,

(a) an amount of an anti-microbial composition effective to reduce or eliminate C *pneumoniae* infection, and

(b) an amount of an anti-viral composition effective to reduce or eliminate HCMV infection

28. Use of a composition according to any of claims 25 to 27 in the preparation of a medicament for the treatment of atherosclerotic lesions or restenosis

29 Use of an immediate-early exon-4 subunit of HCMV in the preparation of a medicament for preventing or retarding the development of atherosclerotic lesions or restenosis in a mammal, wherein said medicament is administered to the mammal in an amount inducing cell mediated immunity and/or antibody response to HCMV in the mammal

STATEMENT UNDER ARTICLE 19

Pursuant to Article 19(1), Applicant has amended the original claims and encloses replacement pages 36, 39 and 41. Claim 1 is amended to eliminate the words "or fragment thereof" in line 2. Claims 23 and 24 are cancelled. Claim 29 is added to claim the use of a specific fragment, that of the IE-exon 4 subunit of HCMV as provided on page 8, lines 33-34.

No changes to the drawings are required by these amendments. Only minor amendments need be made to the specification in view of these amendments, because the eliminated fragments and antibiotic compositions referred to in claims 1 and 23 and 24, are useful in combinations covered by several of the unamended original claims.

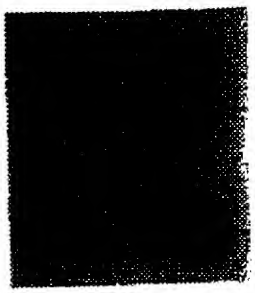

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

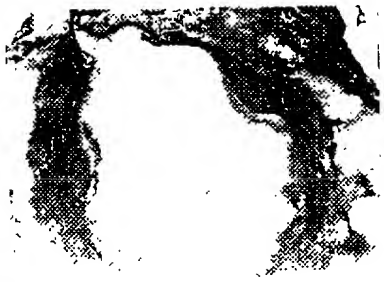

FIG. 1E

FIG. 1F

FIG. 1G

FIG. 1H

FIG. 1I

FIG. 1J

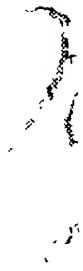

FIG. 1K

FIG. 1L

FIG. 2A FIG. 2B FIG. 2C

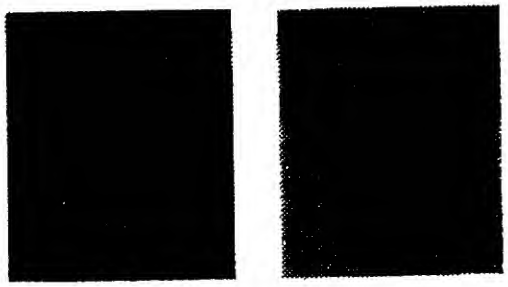

FIG. 3A FIG. 3B

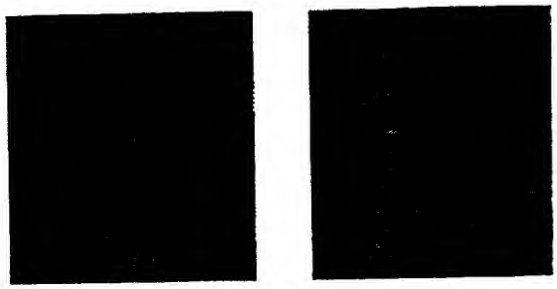

FIG. 4A FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/164.1, 230.1, 263.1; 435/320.1; 514/44; 530/350, 395; 536/23.7, 23.72.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X -- Y	US 5,534,258 A (GOLUBEV et al.) 09 july 1996, see entire document	1-3 ------ 4-9, 16-24
X -- Y	US 5,424,187 A (SHOR et al.) 13 June 1995, see entire document.	23-24 ----------- 1-9, 16-22
Y	MELNICK et al. Possible role of cytomegalovirus in atherogenesis. Journal of the American Medical Association. 25 April 1995, Vol. 263, No. 16, pages 2204-2207, see entire document.	1-9 --------- 16-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 1997	17 NOV 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ROBERT D. BUDENS
Facsimile No (703) 305-3230	Telephone No (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14443

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KUO et al. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. The Journal of Infectious Diseases. 1993, Vol. 167, pages 841-849, see entire document.	1-9, 16-24
Y	SORLIE et al. Cytomegalovirus/herpesvirus and carotid atherosclerosis: The ARIC study. Journal of Medical Virology. 1994, Vol. 42, pages 33-37, see entire document.	1-9, 16-24
Y	MELNICK et al. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. Journal of Medical Virology. 1994, Vol. 42, pages 170-174, see entire document.	1-9, 16-24
Y	KUO et al. Chlamydia pneumoniae (TWAR) in coronary arteries of young adults (15-34 years old). Proceedings of the National Academy of Sciences, USA. 18 July 1995, Vol. 92, pages 6911-6914, see entire document.	1-9, 16-24
Y	MCEWAN, J. Potential for antiviral therapy in the treatment of restenosis after angioplasty. British Heart Journal. 1995, Vol. 73, page 489, see entire document.	1-9, 16-24
Y	GRAULS et al. Initial endothelial injury and cytomegalovirus infection accelerate the development of allograft arteriosclerosis. Transplantation Proceedings. December 1995, Vol. 27, No. 6, pages 3552-3554, see entire document.	1-9, 16-24
Y	GRAYSTON et al. Chlamydia pneumonia (TWAR) in atherosclerosis of the carotid artery. Circulation: In The American Heart Association. 15 December 1995, Vol. 92, No. 12, pages 3397-3400, see entire document.	1-9, 16-24
Y	MLOT, C. Chlamydia linked to atherosclerosis. Science. 07 June 1996, Vol. 272, page 1422, see entire document.	1-9, 16-24
Y	MUHLESTEIN et al. Increased incidence of chlamydia species within the coronary arteries of patients with symptomatic atherosclerotic versus other forms of cardiovascular disease. Journal of the American College of Cardiology. June 1996, Vol. 27, No. 7, pages 1555-1561, see entire document.	1-9, 16-24
Y	ONG et al. Detection and widespread distribution of Chlamydia pneumoniae in the vascular system and its possible implications Journal of Clinical Pathology. 1996. Vol. 49, pages 102-106, see entire document	1-9, 16-24

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14443

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically.

3. ☒ Claims Nos.: 10-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1 ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3 ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-9, 16-24

4 ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest

☐ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14443

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 31/70, 39/118, 39/245, 39/40, 39/42; C12N 15/63; C07K 14/045, 14/295; C07H 21/04.

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/164.1, 230.1, 263.1; 435/320.1; 514/44; 530/350, 395; 536/23.7, 23.72

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-15 drawn to methods of treating atherosclerosis using cytomegalovirus proteins and nucleic acids.
Group II, claim(s) 16-22, drawn to methods of treating atherosclerosis using chlamydial proteins or nucleic acids.
Group III, claim(s) 23-24, drawn to methods of treating atherosclerosis using anti-microbial compositions.
Group IV, claim (s) 25-28, drawn to compositions with anti-microbials and antiviral components and method of use.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The methods of Groups I-IV are directed to methods which differ in their industrial applicability, method steps and reagents and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept. Further , the compositions of Group IV are not utilized by the methods of Groups I-III and therefore are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.

US CLAIMS

1. A method for determining, *in vitro*, predisposition to a venous thromboembolic disease in an individual, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*.

2. The method as claimed in claim 1, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, by assaying the level of anti-Chlamydia antibodies in a biological sample from said individual.

3. A pharmaceutical composition comprising at least one agent active against infection with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, or of at least one agent effective against the inflammatory effects of infection with Chlamydia, in a pharmaceutically acceptable carrier, for preventing and/or treating venous thromboembolic disease.

4. The pharmaceutical composition according to claim 3, wherein said agent active against infection with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, is an antibiotic substance.

5. The pharmaceutical composition according to claim 3, wherein said antibiotic substance active on bacteria of the Chlamydia genus, in particular *Chlamydia pneumoniae*, is chosen from the group consisting of macrolides, tetracyclines, fluoroquinolones and rifampicin.

6. The pharmaceutical composition according to claim 3, wherein said medicinal product is intended to prevent recurrences subsequent to a first venous thrombosis.

7. A method for preventing and/or treating venous thromboembolic disease, wherein a prophylactically or therapeutically effective amount of an agent active on bacteria of the Chlamydia genus, in combination with a pharmaceutically acceptable carrier, is administered to a patient requiring such a treatment.

8. A method according to claim 7, wherein said active agent is an antibiotic agent.

9. A method according to claim 7 intended for the prevention of recurrences subsequent to a first venous thrombosis.

Method for determining predisposition to a venous thromboembolic disease

The present invention is based on the demonstration of a close link between infection with a bacterium of the Chlamydia genus and venous thromboembolic disease.

The invention more particularly relates to a method for determining, *in vitro*, predisposition to a venous thromboembolic disease in an individual, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, and to the use of agents active on bacteria of the Chlamydia genus, for preventing and/or treating a venous thromboembolic disease.

Venous thromboembolic disease is a multifactor disorder with both genetic and acquired risk factors (Rosendaal et al., 1997). Conventional risk factors for venous thrombosis are associated with modification of the vessels or stasis (slowing down, or even stopping of venous blood circulation), due in particular to a trauma, a surgical operation or immobilization. Overall, genetic predisposition to venous thrombosis explains only 40% of cases. Deficiencies in antithrombin, in protein C and in protein S explain in particular 5 to 10% of cases of venous thromboembolic diseases. After having taken into account conventional acquired risk factors and genetic predispositions, at least a third of venous thrombotic episodes remain unexplained.

Currently, the most commonly used treatment in cases of venous thrombosis is treatment based on anticoagulants. However, such a treatment can be envisioned only for a limited period of time, because of dangerous side effects. Prolonged administration of anticoagulants in fact increases the risks of hemorrhages, in particular

cerebral hemorrhages. Consequently, an alternative treatment is sought.

The link between infection with *Chlamydia pneumoniae* and atherosclerosis is well documented. The use of antibiotics in the treatment of cardiovascular diseases, such as myocardial infarction or coronary artery diseases, has also been envisioned (WO 90/00061, WO 98/17280, WO 98/06408, Gibbs et al., 1998). The cardiovascular diseases targeted are, however, diseases of the arterial system.

On the other hand, to date, no datum would suggest the possibility of a link between *Chlamydia pneumoniae* and the pathologies of the venous system. Although an article by Ong et al., published in 1996, reported the detection by PCR (polymerase chain reaction) of *Chlamydia pneumoniae* in an iliac vein on two control individuals *a priori* free of pathological vascular conditions, a subsequent article (Bartels et al., 1998) reported, on the contrary, the absence of this bacterium in the native saphenous veins of patients who had undergone a coronary bypass.

The authors of the present invention have discovered, surprisingly, a close link between infection with a bacterium of the Chlamydia genus and a venous thromboembolic disease.

More particularly, the authors of the invention have shown that high levels of anti-*Chlamydia pneumoniae* antibodies represent a risk factor for a venous thromboembolic disease.

Without in any way associating themselves with a precise mechanism of action, the authors are putting forward the hypothesis that chronic infection of the vein walls with *Chlamydia pneumoniae* may render the venous endothelial cells thrombogenic.

A subject of the present invention is therefore a method for determining, *in vitro*, predisposition to a venous thromboembolic disease in an individual, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, more particularly *Chlamydia pneumoniae*.

It is determined whether the individual has been infected with a bacterium of the Chlamydia genus by analyzing a biological sample. It may in particular be a sample of blood, of urine or of pleural liquid, a sample obtained by bronchoscopy or by bronchoalveolar lavage, or a sample obtained by biopsy, for example of the vascular endothelium. It is then possible to determine whether this sample contains anti-*Chlamydia* antibodies or whether it contains bacteria of the Chlamydia genus or fragments thereof. It is, for example, possible to search for the presence of a chlamydial component, such as liposaccharides or membrane-bound proteins, of substances produced by *Chlamydia*, such as exopolysaccharides, or of substances produced by the host cells via *Chlamydia* induction.

Preferably, it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, by assaying the level of anti-*Chlamydia* antibodies in a biological sample from an individual to be tested, for example a blood sample.

The anti-*Chlamydia* antibody titers obtained in the individuals to be tested are then compared with the antibody titers obtained in control individuals. The titer is defined by the maximum dilution of the biological sample for which the antibodies are still detected, and is expressed by the inverse of the dilution factor. An antibody titer of greater than 256 may be considered to represent a not insignificant risk factor.

A subject of the present invention is therefore, more particularly, a method for determining predisposition to a venous thromboembolic disease in an individual, comprising the steps consisting in:

i) assaying the level of anti-chlamydia antibodies in a biological sample from an individual to be tested;

ii) comparing this level of antibodies with the level of anti-chlamydia antibodies obtained in control individuals;

iii) identifying the individual tested as an individual exhibiting predisposition to a venous thromboembolic disease if the level of antibodies obtained in step i) is greater than the level of anti-chlamydia antibodies obtained in control individuals.

The present invention also relates to the use of at least one agent active against infection with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, or of at least one agent effective against the inflammatory effects of infection with *Chlamydia*, for preventing and/or treating a venous thromboembolic disease.

Preferably, pharmaceutical compositions containing antibiotic agents may be used.

A subject of the invention is therefore more particularly the use of an antibiotic substance active on bacteria of the Chlamydia genus, in particular *Chlamydia pneumoniae*, for preparing a medicinal product intended to prevent and/or treat a venous thromboembolic disease.

Among the antibiotic substances or agents active on the bacterial genus Chlamydia, mention may in particular be made of macrolides (for example erythromycin or azithromycin), tetracyclines, fluoroquinolones and

rifampicin.

According to another embodiment of the present invention, said agent active against infection with *Chlamydia* may be an immunogenic protein, or an immunogenic fragment of a protein, of *Chlamydia*, in particular of *Chlamydia pneumoniae*. These proteins, or these protein fragments, are characterized by their ability to induce cell-mediated or humoral immunity against infection with *Chlamydia*, in particular *Chlamydia pneumoniae*, by administration of the protein in combination with a suitable adjuvant. Preferably, use may be made of the major outer membrane protein or cell surface proteins from the bacterium, which may be randomly fragmented. The random fragments of a *Chlamydia* protein can be tested for their immunogenicity by those skilled in the art. Vaccines containing cellular antigens of Chlamydia [lacuna] fragments thereof may be obtained conventionally, for example by cell lysis or using standard purification or separation techniques.

A composition which can be used according to the invention may also comprise a *Chlamydia* bacterium which has been killed or inactivated by any conventional means, such as heat or irradiation.

A composition which can be used according to the present invention may also contain one or more nucleic acid sequences encoding a surface protein of *Chlamydia* or a fragment thereof. The nucleic acid used may be administered using an immunization vector or in naked form, i.e. free of any agent which facilitates the penetration of this nucleic acid into the cell.

A subject of the invention is also a method for preventing and/or treating venous thromboembolic disease, in which a prophylactically or therapeutically effective amount of an agent active on bacteria of the

Chlamydia genus, in particular of an antibiotic agent, in combination with a pharmaceutically acceptable vehicle, is administered to a patient requiring such a treatment.

The prevention of recurrences subsequent to a first venous thrombosis is more particularly targeted.

The methods of an administration, the doses and the pharmaceutical forms of the pharmaceutical compositions which can be used according to the invention may be determined in the usual way by those skilled in the art, in particular according to the criteria generally taken into account in establishing a therapeutic treatment suitable for a patient, such as for example the age or body weight of the patient, the seriousness of his or her general condition, the tolerance to the treatment and the side effects noted, etc.

A pharmaceutical composition which can be used according to the invention may in particular be administered orally, parenterally, intravenously, intramuscularly, subcutaneously, percutaneously or intranasally.

When the pharmaceutical composition used is an antibiotic composition, the effective dose lies within the ranges conventionally used for any antibiotic against Chlamydia bacteria. Said composition may in particular be advantageously administered in short cycles (4 to 10 days approximately), to be repeated, for example, every six months after the first venous thrombotic episode.

The link between infection with a *Chlamydia pneumoniae* bacterium and a venous thromboembolic disease is illustrated in the results presented hereinafter, which in no way limit the scope of the present invention.

The authors of the present invention have thus studied 176 patients with a diagnosed venous thromboembolic disease and 197 control individuals in good health, of various age and sex. The acquired risk factors for a venous thromboembolic disease and the common genetic predisposition factors (Arg 506 Gln mutation in factor V and G 20210 A mutation in factor II) were evaluated in all the individuals. The level of anti-*C. pneumoniae* IgG antibodies was determined by microimmunofluorescence. All the positive plasma samples (titer ≥ 128) were accurately quantified and tested for the presence of specific IgM antibodies.

MATERIALS AND METHODS:

Individuals:

The patients less than 61 years old who had had at least one episode of deep vein thrombosis diagnosed objectively (compression ultrasonography or venography) and/or a pulmonary embolism (ventilation and perfusion pulmonary scintigraphy, conventional pulmonary angiography and a spiral scan) were selected from 205 patients (92 men and 113 women) with venous thromboembolic diseases. A complete clinical study was carried out on all the patients, emphasizing their personal and family histories regarding thromboembolic disease, and the acquired risk factors (surgery or trauma within the last three months, immobilization for longer than 72 hours, pregnancy, treatment with estrogens, varicose veins and cancer). Blood was taken from 176 patients (86%); in 87 cases, samples were obtained less than three months after an acute venous thromboembolic disease had been triggered (median 1 day; IQR deviation 0-16), whereas the remaining 89 samples were obtained more than three months after triggering (median 12 months, IQR deviation 6.5-36). The 197 healthy control individuals of varied age and sex were selected as having no history of venous

thromboembolic disease, of myocardial infarction or of peripheral vascular disease.

Assay:

The venous blood was collected in 0.129 M trisodium citrate (1:10) and two steps of centrifugation at 2000 g for 15 minutes were carried out in order to obtain a platelet-depleted plasma. The plasma was frozen and stored in aliquot fraction form at -40°C until use. The DNA was extracted from the leukocytes using standard methods and stored at 4°C.

For the serological tests, each case and each control sample were labeled with a random number and analyzed blind. The serological status in terms of *C. pneumoniae* was determined by a microimmunofluorescence (MIF) assay using the "SeroFIA IgG Chlamydia" kit (Savyon Diagnostics Ltd., Ashdod, Israel). Briefly, elementary bodies of purified *C. pneumoniae* (IOL 207 strain) were used to detect the IgG antibodies. All the plasma samples were initially screened at a dilution of 1:128 and were considered to be positive above this dilution. The positive plasmas were then tested at dilutions of 1:256, 1:512 and 1:1024. The specific IgG titers were given as the inverse of the final positive dilution. Samples positive for anti-*C. pneumoniae* IgGs were then tested for the presence of anti-*C. pneumoniae* IgMs using the MIF assay with the "SeroFIA IgM Chlamydia" kit (Savyon Diagnostics Ltd), at a dilution of 1:20 as recommended by the manufacturer.

A study was, moreover, carried out to determine whether the DNA of the control individuals and of the patients exhibited the Arg 506 Gln mutation in factor V, after PCR amplification of exon 10 of factor V and digestion with restriction enzymes. The transition G 20210 A of the prothrombin gene was identified after amplification using two primers:

(5'-TTACAAGCCTGATGAAGGGA-3'
and 5'-CCATGAATAGCACTGGGAGCATTGAAGC-3'). The second primer was constructed such that a nucleotide substitution (C to A) at position 20210 creates a new restriction site for *Hind III* when the transition of G to A at position 20210 is present.

Statistical analysis:

The data are analyzed using the SAS statistical program (Institute Inc., Cary, N.C.). The clinical characteristics of the entire population of patients and of the subgroup of cases with blood samples taken less than three months after the episode of thrombosis were compared to those of the control individuals using a Chi^2 test with one degree of freedom. Age was tested using analysis of variance (ANOVA).

The odds ratios associated with seropositivity for *C. pneumoniae* were calculated by comparing the individuals having titers of 256 or more having individuals with titers of less than 256. The heterogeneity of the odds ratios in terms of age and sex was tested by entering the interaction variables (one degree of freedom) into logistic regressions. The odds ratios for a venous thromboembolic disease and the 95% confidence interval (CI) associated with each anti-*C. pneumoniae* IgG titer, coded as binary variables, were then calculated with reference to seronegativity using a logistic regression procedure (SAS-PROC LOGIST). The same analysis was applied to the subgroup of cases with blood samples taken less than three months after the venous thromboembolic disease had been triggered. In the entire population tested, the levels of seropositivity for *C. pneumoniae* were compared in terms of first thrombosis against recurrent thrombosis, in terms of presence of associated risk factor against absence of associated risk factor, and in terms of first thrombosis at under 40 years of age (median age of the

population) against first thrombosis at over 40 years of age, using a Chi^2 test with one degree of freedom. The differences with values p less than 0.05 were considered to be significant.

RESULTS:

The cases and controls do not differ in terms of age ratios or sex ratios (cf. table 1). Venous thromboembolic disease was considered to be idiopathic in patients who had not been taking any oral contraception, who had not recently had surgery (less than one month), who had not suffered any trauma, who had not been pregnant or given birth, who had not been immobilized or who had not suffered from cancer. Half of the patients were recruited within three months after the acute episode of venous thrombosis. The characteristics of this subgroup were not statistically different from those of the entire population of cases. The prevalence of the Arg 506 Gln mutation of factor V and the G 20210 A mutation of factor II was within the range expected in caucasians (21.9% in the patients and 5.1% in the controls, $p<0.0001$; and 10.2% and 4.1%, $p=0.02$, respectively).

The *C. pneumoniae*-specific IgG titers tend to be higher in the patients than in the controls (cf. table 2). Significantly, it is observed that more patients than controls have *C. pneumoniae* IgG titers of 256 or more (54% and 15.9% respectively, $p<0.0001$). The odds ratio for the venous thromboembolic diseases associated with IgG titers of 256 or more was 6.2 (95% confidence interval (CI), 3.8-10.1). In the subgroup of patients with blood samples taken less than three months after the thrombotic episode, the crude ratio (odds ratio) among the individuals with IgG titers ≥256 was 5.4 (95% CI, 3.1-9.6). In addition, the odds ratio for a venous thromboembolic disease increases with the IgG titer: for titers of 256, 512 and 1024, the crude odds ratios

were 2.1 (95% CI, 1.0-4.2), 4.3 (2.1-8.9) and 32.4 (4.2-248.3), respectively (cf. table 3). A greater proportion of seropositive controls had a low IgG titer of 128 compared to the controls (54.9% and 21%, respectively). Similar odds ratios were obtained in the subgroup of cases tested within three months after the thrombotic episode.

The odds ratios were not significantly different according to age or sex. After exclusion of the individuals bearing the Arg 506 Gln mutation of factor V and the G 20210 A mutation of factor II, the odds ratios for the venous thromboembolic disease associated with a titer greater than 256 was 7.7 (95% CI, 4.5-13.2). The characteristics of the venous thromboembolic disease (age at the time of the first episode; recurrent or spontaneous nature) did not differ as a function of the serological status for *C. pneumoniae*.

In order to distinguish an acute infection from a chronic infection, the authors of the invention also evaluated the circulating anti-*C. pneumoniae* IgM antibodies in the 95 cases and 31 controls who had IgG titers greater than 256. Only one individual from the group of patients suffering from venous thromboembolic diseases was positive for IgMs.

CONCLUSION:

The results above clearly show a link between the serological status for *C. pneumoniae* and venous thrombosis. The odds ratio for a venous thromboembolic disease associated with circulating titers of anti-*C. pneumoniae* IgG antibodies of 256 or more was 6.2 (95% CI; 3.8-10.1) and remained high after exclusion of the patients with an Arg 506 Gln mutation in factor V and a G 20210 A mutation in factor II (odds ratio 7.7; 95% CI; 4.5-13.2). The fact that the odds ratio for a venous thromboembolic disease increases with the IgG

antibody titer reinforces this association (cf. table 3).

	Cases n=176	**Controls n=197**	**Test**
% women	56.2	55.3	p=0.87
Mean age (SD)	42.8(10.7)	42.9(10.6)	p=0.92
% oral contraception in women	34.0	22.0	p=0.053
% FV mutation-Arg 506Gln	21.9	5.1	p<0.0001
% FII mutation-G20210A	10.2	4.1	p=0.02
% spontaneous TED	37.6	-	-
% recurrent TED	26.7	-	-
% pulmonary embolism	37.1	-	-
Mean age at first TED (SD)	38.4 (12.0)	-	-

TED: venous thromboembolic disease

SD: standard deviation

Table 1: **Characteristics of the cases with a venous thromboembolic disease (TED) and of the controls.**

C. pneumoniae **IgG titers**	**Controls n(%)**	**All cases n(%)**	**OR(95% CI)**	**Cases < 3 months*** **n(%)**	**OR(95% CI)**
Negative	57(29.2)	44(25.0)		26(29.9)	
128	107(54.9)	37(21.0)		17(19.5)	
256	17(8.7)	27(15.4)		14(16.1)	
			6.2(3.8-10.1)**		5.4(3.1-9.6)**
512	13(6.7)	43(24.4)	p<0.0001	20(23.0)	p<0.0001
1024	1(0.5)	25(14.2)		10(11.5)	

*Cases with blood samples taken within three months after the TED episode.

** <256 vs ≥256

CI: confidence interval

OR: odds ratio

Table 2: **Detection and titration of anti-*Chlamydia pneumoniae* IgG antibodies in the cases and controls, and odds ratios (95% CI) for a venous thromboembolic disease (TED) associated with IgG titers ≥256**

C. pneumoniae **IgG titers**	**All cases**			**Cases < 3 months***		
	OR	**(95% CI)**	**p**	**OR**	**(95% CI)**	**p**
Negative	1	-	-	1	-	-
128	0.4	(0.3-0.8)	0.004	0.3	(0.2-0.7)	0.0028
256	2.1	(1.0-4.2)	0.05	1.8	(0.8-4.2)	0.17
512	4.3	(2.1-8.9)	<0.0001	3.4	(1.5-7.8)	0.0045
1024	32.4	(4.2-248.3)	0.0008	21.9	(2.7-180.3)	0.0041

*Cases with blood samples taken within three months after the TED episode

CI: confidence interval

OR: odds ratio

Table 3: **Odds ratios (95% CI) for a venous thromboembolic disease (TED) as a function of the anti-*Chlamydia pneumoniae* IgG titers with reference to the absence of detectable antibodies.**

REFERENCES

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CLAIMS

1. A method for determining, *in vitro*, predisposition to a venous thromboembolic disease in an individual, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*.

2. The method as claimed in claim 1, in which it is determined whether the individual has been infected with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, by assaying the level of anti-Chlamydia antibodies in a biological sample from said individual.

3. The use of at least one agent active against infection with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, or of at least one agent effective against the inflammatory effects of infection with Chlamydia, for preparing a medicinal product intended to prevent and/or treat venous thromboembolic disease.

4. The use as claimed in claim 3, in which said agent active against infection with a bacterium of the Chlamydia genus, in particular *Chlamydia pneumoniae*, is an antibiotic substance.

5. The use as claimed in claim 4, in which said antibiotic substance active on bacteria of the Chlamydia genus, in particular *Chlamydia pneumoniae*, is chosen from the group consisting of macrolides, tetracyclines, fluoroquinolones and rifampicin.

6. The use as claimed in any one of claims 3 to 5, in which said medicinal product is intended to

prevent recurrences subsequent to a first venous thrombosis.

Rec'd PCT/PTO 19 NOV 2001

DECLARATION FOR USA PATENT APPLICATION

(including Design and National Stage PCT)

Attorney's Docket ID: 09/937035

As a below named inventor, I hereby declare that:
My residence, mailing address and citizenship are as stated below adjacent to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought

on the invention entitled: Method for determining predisposition to a venous thromboembolic disease

the specification of which:

___ is attached hereto.
(or)
___ was filed on March 22, 2000 as U.S. Application No. or PCT International Application No. PCT/FR0000725

and (if applicable) was amended on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below, where priority is not claimed, any foreign application for patent or inventor's certificate, or any PCT International application, having a filing date before that of the application on which priority is claimed. (__ ADDITIONAL APPLICATIONS IDENTIFIED ON ATTACHED SHEET)

Prior Foreign Application No.	Country	Day/Month/Year Filed	Priority Not Claimed
99 03613	FRANCE	23/03/99	YES

I hereby claim the benefit under 35 U.S.C. 120 of any U.S. application(s), or 365(c) of any PCT application designating the U.S., listed below; and insofar as the subject matter of each claims of this application is not disclosed in the prior U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT filing date of this application. (___ ADDITIONAL APPLICATIONS IDENTIFIED ON ATTACHED SHEET.)

U.S. or PCT Parent Application No.	Parent Filing Date (Day/Month/Year)	Parent Patent No. (if applicable)

As a named inventor, I hereby appoint the registered practitioners of **LARSON & TAYLOR, PLC** associated with **Customer Number 000881** to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to that Customer Number.

Direct all telephone calls to _____
at TEL (703) 739-4900 (Fax. 703-739-9577) e-mail:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1000 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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